Effect of Sodium Chloride on Uptake of Substrate by *Staphylococcus aureus* 196E

ABSTRACT

Sodium chloride inhibited a number of biochemical parameters in *Staphylococcus aureus* 196E. Induction of phospho-ß-galactosidase, synthesis of staphylococcal enterotoxin A, enzyme activity (phospho-ß-galactosidase) and glucose utilization were approximately four times more sensitive to the inhibitory effects of salt than was growth. Uptake of ^{14}C-2-deoxyglucose and respiratory activity with a number of substrates were inhibited also. The breakdown of o-nitrophenyl-ß-galactoside (ONPG) by lactose-grown *S. aureus* 196E was inhibited by NaCl as well as by other solutes (salts, carbohydrates, amino acids) which suggested that the inhibitory effect is a general one of solutes and not restricted to NaCl. Various ionophores (gramicidin, valinomycin, monensin, lasalocid, m-chlorophenylhydrazone), the H^+-ATPase inhibitor (N,N',-dicyclohexylcarbodiimide), and ion channel blockers (quinine, quinidine, chlorpromazine, tetracaine, verapamil) reversed the inhibitory action of salt on ONPG breakdown by lactose-grown cells; however, these compounds did not reverse NaCl inhibition of glucose utilization. The effects observed here suggest that NaCl (and probably other solutes) exerts an inhibitory effect on transport of substrates into the cells of *S. aureus* 196E.

Sodium chloride plays many roles when it is used as an additive in food products. It acts as a flavoring agent and it has profound effects on functional properties of various foods. Additionally, NaCl acts as an antimicrobial agent by inhibiting growth and/or activity of spoilage and pathogenic microorganisms. The exact mechanism by which salt exerts its antimicrobial effects is not well understood.

Sofos *(22)* suggested that the inhibitory action of NaCl in foods was indirect because addition of salt lowered the water activity (a_W) with concomitant decrease in the growth and biochemical activities of many spoilage and pathogenic bacteria. Thus inhibition is due to a non-specific removal of water from the microbial environment. Other workers presented data that suggest that NaCl may have more direct effects not dependent on decrease in a_W per se. Using concentrated cell suspensions of *Clostridium sporogenes,* Woods and Wood *(26)* demonstrated that there was a progressive decrease in glucose utilization and loss of intracellular ATP (over a 90-min period) with increasing concentrations of salt. Erecinska and Deutsch *(5),* using washed cells of *Paracoccus dentrificans,* found that the rate of respiration and uptake of α-aminoisobutyrate decreased progressively with the increase of NaCl levels. From these findings, it would appear that NaCl interferes with substrate utilization in some manner.

In this study, attention was focused on the ability of *Staphylococcus aureus* 196E to utilize various substrates in the presence of NaCl to gain some understanding of the mode of antibacterial action of NaCl.

MATERIALS AND METHODS

Microorganism and chemicals

S. aureus 196E was obtained from the Eastern Regional Research Center (Philadelphia, PA) stock culture collection. The culture was maintained frozen (-20°C) in tryptic soy broth (Difco); a tube of frozen culture was thawed and incubated overnight at 37°C before its use as an inoculum. Glucose and Na lasalocid were obtained from J. T. Baker Chemical Co. and Aldrich Chemical Co., respectively. All other chemicals and reagents were obtained from Sigma Chemical Co.

Effect of NaCl on the growth of S. aureus *196E*

S. aureus 196E at a level of approximately 1×10^5 cells/ml, was inoculated into tryptic soy broth without glucose (Difco; TSB w/o glucose) containing 0 to 10% (w/v) NaCl (since addition of NaCl reduced the pH of the broth, all flasks containing NaCl were adjusted to pH 7.2 with NaOH before autoclaving). Inoculated flasks were incubated on a rotary shaker (200 rpm) at 37°C. At intervals, a sample of culture fluid was removed from each flask and diluted into sterile 0.1% peptone (Difco) water. Appropriate dilutions were plated on tryptic soy agar (Difco; TSA) utilizing a spiral plater (Spiral System Instruments, Inc., Bethesda, MD). Plates were incubated at 37°C for 48 h before counting.

Effect of NaCl on staphylococcal enterotoxin A (SEA) production

S. aureus 196E was grown at 37°C for 16 h in TSB w/o glucose on a rotary shaker (200 rpm). The cells were harvested by centrifugation and washed 2× with sterile distilled water. The cells were added at a level of approximately 1×10^9 cells/ml to the enterotoxin production medium which was a casamino

acids salts medium (CAS; *21*) containing 0 to 10% (w/v) NaCl (CAS was adjusted to pH 7.0 with NaOH after addition of NaCl). At 24 h, cells were removed from CAS by centrifugation and the supernatant fluid was treated with normal rabbit serum to remove Protein A *(21)*. SEA was determined on the Protein A free supernatant fluids by the ELISA method described by Smith and Bencivengo *(20)*.

Effect of NaCl on phospho-ß-galactosidase induction

S. aureus 196E was grown in TSB w/o glucose containing 1% (w/v) glucose (sterile glucose was added aseptically) for 16 h at 37°C utilizing a rotary shaker (200 rpm). Cells were harvested by centrifugation, washed with sterile distilled water and then added to CAS at a level of approximately 1×10^9 cells/ml. The CAS contained 0.25% (w/v) lactose plus NaCl ranging from 0 to 10% (w/v) (CAS containing NaCl was adjusted to pH 7.0 with NaOH). CAS flasks were incubated on a rotary shaker (200 rpm) at 37°C; at the end of 4 h, cells were harvested by centrifugation, washed twice with sterile distilled water and then assayed for phospho-ß-galactosidase by the method of Dobrogosz *(4)*.

Effect of NaCl on phospho-ß-galactosidase activity

S. aureus 196E was grown 16 h at 37°C in TSB w/o glucose containing 1% (w/v) lactose (sterile lactose was added aseptically) using a rotary shaker (200 rpm). Cells were harvested by centrifugation, washed 2× in sterile distilled water, and concentrated to 1/5 of the original volume in sterile distilled water. Portions of cells were used for dry weight determinations (100°C overnight) and for phospho-ß-galactosidase determinations according to the method of Dobrogosz *(4)*. The buffer used for the assay was 0.1 *M* potassium phosphate buffer (pH 7.5) or 0.1 *M* potassium phosphate buffer containing NaCl (pH 7.5). The dry weight of cells added to each assay tube was 8 to 10 mg.

In some experiments, o-nitrophenyl-ß-galactoside-6-phosphate (ONPG-P) was used as the substrate for phospho-ß-galactosidase rather than o-nitrophenyl-ß-galactoside (ONPG). For these experiments, washed cells of *S. aureus* 196E (grown as described above) were suspended in 0.1 *M* potassium phosphate buffer, pH 7.5 rather than in distilled water. One mg of lysostaphin (Sigma) was added to 10 ml of cell suspension and the mixture was incubated at 37°C for 30 min. One ml of the treated cell suspension (equivalent to 8 to 10 mg of dry weight cells) was added to each assay tube.

Effect of NaCl on respiration

S. aureus 196E was grown in TSB w/o glucose for 16 h on a rotary shaker (200 rpm) at 37°C. When lactose or mannitol utilization was studied, sterile lactose or mannitol, respectively, were aseptically added to TSB w/o glucose to give a final concentration of 1% (w/v). At the end of the incubation period, cells were harvested by centrifugation, washed twice with sterile distilled water, and concentrated to 1/5 of the original volume in distilled water. Portions of the washed cells were used for dry weight determinations (100°C overnight) and respirometry. Single sidearm flasks (15 ml) containing 3 ml were used. Each flask contained potassium phosphate buffer (0.1 *M*, pH 7.3) or 0.1 *M* buffer containing 5% NaCl (pH 7.3), and washed cells (4-5 mg of dry weight) in the main compartment; 0.2 ml 20% (w/v) KOH in the center well (with folded filter paper); and 0.01 *M* substrate (in buffer and adjusted if necessary to pH 7.3 with NaOH) in the sidearm. The flasks were attached to a differential respirometer (Gilson Model IG-20, Middleton, WI) and equilibrated to 37°C. The substrate was then tipped in and the O_2 uptake and CO_2 production monitored. All respirometric values were corrected for endogenous activity.

Effect of NaCl on glucose utilization and ^{14}C-2-deoxyglucose (2-DOG) uptake

S. aureus 196E was grown for 16 h at 37°C in TSB w/o glucose on a rotary shaker (200 rpm). Cells were harvested by centrifugation, washed 2× with sterile distilled water, and resuspended in 1/30 of the original volume in distilled water. A portion of cells was used for dry weight determination (100°C overnight); the remainder was used to inoculate the glucose assay medium. The glucose assay medium was tryptose phosphate broth (TPB, contains 0.2% glucose; Difco) with or without NaCl (the pH of TPB was adjusted to 7.3 after NaCl was added). Each flask received 1 ml of inoculum (approximately 40 mg of dry weight) and were incubated with shaking (200 rpm) for 120 min at 37°C. At intervals, samples were taken and cells removed by centrifugation. The supernatant fluids were assayed for glucose by the o-toluidine reagent method (Sigma Diagnostics).

To study 2-DOG uptake, a portion of cells (see above) was inoculated into TSB w/o glucose containing 0.25% 2-DOG (w/v) plus 1.6 µCi uniformly labeled ^{14}C-2-DOG (Amersham Corp.) with or without 5% (w/v) NaCl (TSB w/o glucose containing NaCl was adjusted to pH 7.3). Flasks were incubated with shaking (200 rpm) at 37°C for 120 min. At intervals, 1-ml samples were removed, cells collected by centrifugation, washed 3× with distilled water, and then resuspended in 1 ml of distilled water. The washed cell suspension was added to 10 ml of scintillation fluid (Scinti Verse™ II; Fisher Scientific Co.) for counting with a Beckman Liquid Scintillation Spectrophotometer.

Effect of ionophores, ATPase inhibitors, and ion channel blockers

In some experiments, ionophores, ATPase inhibitors, and ion channel blockers were tested to determine their effect on cell viability, phospho-ß-galactosidase, and substrate uptake. Na_3VO_4, tetraethylammonium chloride, quinine HCl, quinidine HCl, chlorpromazine HCl, tetracaine HCl, and verapamil HCl were water soluble. However, gramicidin, valinomycin, monensin Na, lasalocid Na, carbonylcyanide m-clorophenylhydrazone (CCCP), and N,N'-dicyclohexylcarbodiimide (DCCD) were not water-soluble and were dissolved in 95% ethanol; an ethanol control was always included in each experimental run with these alcohol soluble compounds.

Data

Each experiment was replicated at least 3 times. The data shown in each figure or table represents a typical experiment.

RESULTS

Sodium chloride inhibited a number of cellular activities in *S. aureus* 196E. The growth of staphylococci was less affected by NaCl than certain other biochemical parameters such as SEA synthesis or induction of phospho-ß-galactosidase, enzyme activity (phospho-ß-galactosidase), or utilization of glucose (Fig. 1). The data in Fig. 1 represent best fit straight lines calculated by regression analysis. The slope of the line for salt inhibition

of cell growth (-2.416) was considerably less negative than the slopes for the other parameters (-8.511 to -9.795). Thus SEA production, induction and enzymatic activity of phospho-ß-galactosidase, and glucose utilization were 3.5 to 4.1 times more sensitive to salt than was growth.

Sodium chloride at 5% concentration inhibited oxygen uptake and carbon dioxide production in washed cells of *S. aureus* 196E utilizing sugars, polyols, glutamate, or succinate (Table 1). Respiration on succinate was completely abolished by 5% NaCl.

Not only did NaCl inhibit the induction of phospho-ß-galactosidase (with lactose as the inducer) by glucose-grown cells of *S. aureus* (Fig. 1) but salt also inhibited the breakdown of o-nitrophenyl-ß-galactoside (ONPG; a substrate of phospho-ß-galactosidase utilized by intact cells) by lactose-grown staphylococci (Fig. 1). The assay for phospho-ß-galactosidase is a convenient marker and therefore, we tested the effect of other salts and solutes on the activity of the enzyme. Sodium, potassium, and ammonium salts (at 0.86 *M;* equivalent to 5% NaCl) inhibited enzymatic activity (Table 2). Sugars, polyols, amino acids and NaCl tested at a_w 0.950 also inhibited the breakdown on ONPG by phospho-ß-galactosidase (Table 2). Inhibition of substrate utilization (here ONPG) by solutes may be a general phenomenon.

Since the inhibitory effect of NaCl may be related to the transport of ions, the effect of various ionophores, i.e., compounds which increase the permeability of ions (gramicidin, valinomycin, monensin, lasalocid, carbonyl-cyanide m-chlorophenylhydrazone), ion channel blockers (quinine, quinidine, chloropromazine, tetracaine, verapamil, tetraethylammonium chloride) and ATPase inhibitors (Na_3VO_4; N,N'-dicyclohexylcarbodiimide) were tested for their effects on the activity of phospho-ß-galactosidase in lactose-grown *S. aureus* 196E. All of the ionophores, gramicidin (an ionophore for H^+ and K^+), valinomycin (K^+), monensin (Na^+), lasalocid (K^+), and CCCP (H^+) and the H^+-ATPase inhibitor, DCCD, reversed the inhibitory action of sodium chloride on phospho-ß-galactosidase activity of *S. aureus* 196E (Fig 2A). Gramicidin, valinomycin, CCCP, and DCCD also stimulated enzyme activity in the absence of NaCl whereas monensin and lasalocid had no effect in the absense of NaCl (Fig. 2A). The ionophores utilized affect either proton, sodium, or potassium transport systems. Since all of them reversed the inhibitory action of salt, there does not appear to be any relationship between reversal of salt inhibition and the type of cation transported by the ionophore.

TABLE 1. *Effect of sodium chloride on the respiration of* S. aureus *196E.*

	µl gas/min/10 mg dry weight cells			
	Buffer alone		Buffer + 5% NaCl	
Substrate	O_2 uptake	CO_2 production	O_2 uptake	CO_2 production
Glucose	16.05	11.65	10.25	6.22
Lactose	11.54	8.64	6.96	4.84
Sucrose	15.09	10.31	9.13	5.07
Maltose	15.82	9.90	6.24	1.61
Glycerol	18.88	6.87	13.01	5.92
Mannitol	10.51	5.52	6.36	2.66
Glutamate	6.45	3.03	1.58	0.68
Succinate	7.41	2.77	0.0	0.0

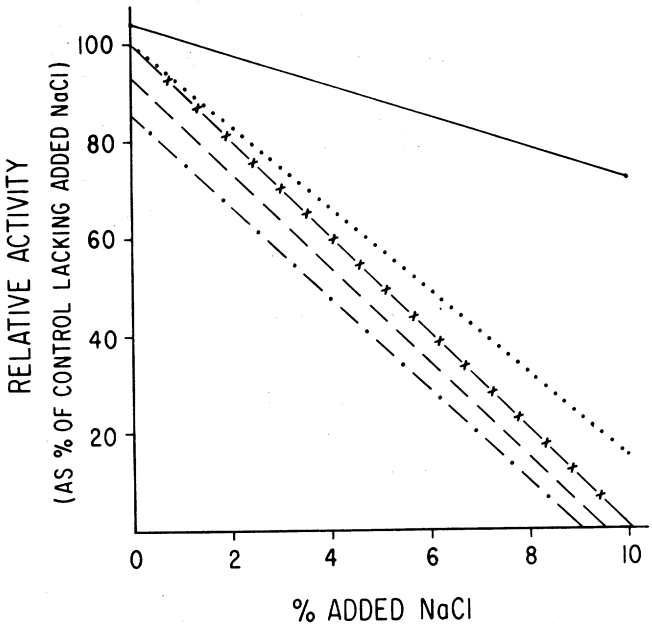
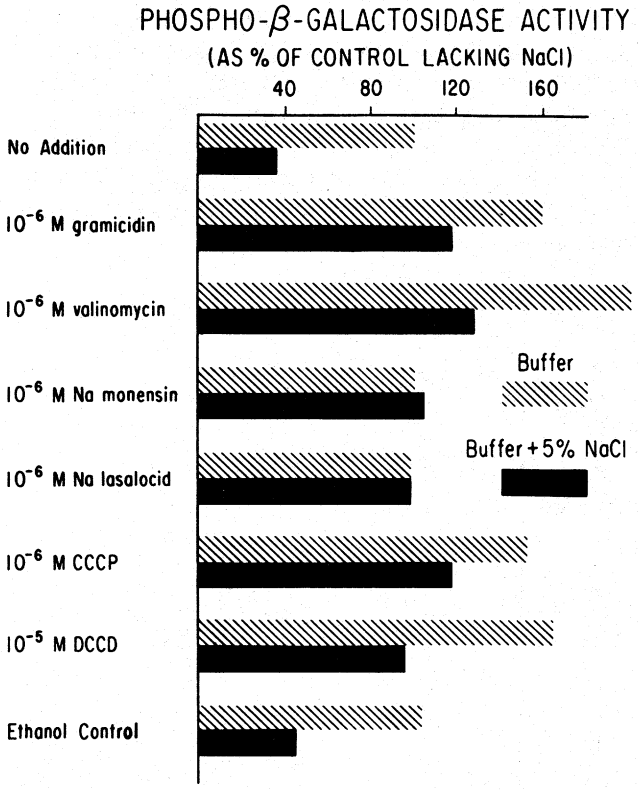


Figure 1. *Effect of varying concentrations of NaCl on selected parameters in* S. aureus *196E.* ———— *growth rate (log_{10} increase in cell numbers/h),* — — — *SEA production (ng/ml),* ..... *induction of phospho-ß-galactosidase activity (µg o-nitrophenol/30 min/10 mg of dry weight cells),* —.—.—. *phospho-ß-galactosidase activity by lactose-induced cells (µg glucose/30 min/10 mg of dry weight cells),* —X—X—X— *glucose utilization (µg glucose/10 min/10 mg of dry weight cells).*

TABLE 2. *Effect of various solutes on phospho-ß-galactosidase activity of lactose-grown* S. aureus *196E.*

Addition to assay buffer[a]	Per cent inhibition of phospho-ß-galactosidase activity
None	0.0
0.86 *M* NaCl	67.7
8.86 *M* Na_2SO_4	41.0
0.86 *M* KCl	73.7
0.86 *M* KNO_3	79.2
0.86 *M* NH_4Cl	46.9
0.86 *M* $(NH_4)_2SO_4$	15.1
None	0.0
NaCl[b]	90.2
Glucose	94.4
Sucrose	62.2
Glycerol	59.9
Sorbitol	99.7
ß-Alanine	68.7
L-Proline	88.1

[a]Potassium phosphate buffer, pH 7.5, 0.01 *M*.
[b]NaCl, sugars, polyols and amino acids were added at a level to give a_w of 0.950 as calculated by the methods of Troller and Christian *(24)* and Chirife et al. *(2,3)*.



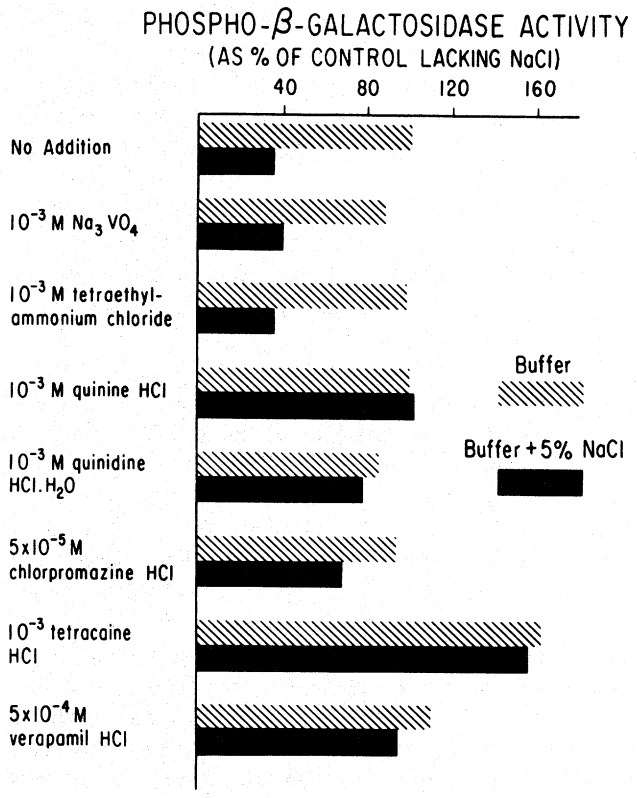


Figure 2, A (left) and B (right). *Effect of ionophores, ATPase inhibitors, and ion channel inhibitors, in the presence or absence of 5% NaCl, on the activity of phospho-ß-galactosidase of lactose-grown* S. aureus *196E.*

The results presented in Fig. 2A suggest that the ionophores and DCCD overcame the NaCl inhibition of substrate (ONPG) uptake by increasing the permeability of the cells to ONPG. Additional experiments were performed to determine if the ionophore, monensin, increased permeability of cells on ONPG. Sodium chloride inhibited induction of phospho-ß-galactosidase (Fig. 1) and it can be postulated that salt prevents uptake of the inducer, lactose. If ionophores overcome salt inhibition by increasing permeability of the cells to ONPG, then addition of an ionophore to cells grown in glucose and then exposed to lactose and NaCl should allow enzyme synthesis. However, the data in Table 3 indicate that monensin does not reverse inhibition of phospho-ß-galactosidase synthesis by 5% NaCl but actually can inhibit enzyme induction in the absence of salt. Therefore, permeability effects do not appear to be an explanation for the ability of ionophores and DCCD to reverse the inhibitory effects of NaCl on enzyme activity.

The ion channel blockers, quinine (blocks K^+ channels), quinidine (Na^+), chlorpromazine (Ca^{++}), tetracaine (Ca^{++}) and verapamil (Ca^{++}), similar to the ionophores and DCCD, reversed the inhibitory action of 5% NaCl on phospho-ß-galactosidase activity (Fig. 2B). However, the ATPase inhibitor Na_3VO_4, and the potassium channel inhibitor, tetraethylammonium chloride had no effect. Only tetracaine stimulated enzyme activity in the absence of salt (Fig. 2B). The compounds that reversed salt inhibition of enzyme activity represented potassium, sodium, and calcium ion channel blockers and thus there appears to be no relationship between the type of ion channels inhibited and the reversal of the salt effect.

TABLE 3. *Effect of monensin on induction of phospho-ß-galactosidase in the presence or absence of NaCl by* S. aureus *196E.*

Addition	Phospho-ß-galactosidase activity	
	CAS-lactose[a]	CAS-lactose-NaCl[a]
None	129.3[b]	38.8
10^{-5} *M* monensin Na	12.4	16.1
10^{-6} *M* monensin Na	14.5	17.1
10^{-7} *M* monensin Na	85.4	21.8
Ethanol control[c]	110.5	54.2

[a]Washed cells of glucose-grown *S. aureus* 196E were added to CAS medium containing 0.5% (w/v) lactose (inducer) with or without 5% (w/v) NaCl.
[b]Enzyme activity expressed as: μg o-nitrophenol released from ONPG in 30 min by 10 mg dry weight cells.
[c]Monensin Na was dissolved in 95% ethanol.

The data in Table 4 indicate that gramicidin, Na_3VO_4, quinine, and tetracaine were strongly lethal to *S. aureus* 196E under the phospho-ß-galactosidase assay conditions; chlorpromazine had a slight effect (approximately one log reduction in cell numbers). The other compounds had no lethal effect. There was no clear cut relationship between lethality and reversal of NaCl inhibition (Fig. 2A and 2B).

TABLE 4. *Effect of ionophores, ATPase inhibitors, and ion channel blockers on the viability of* S. aureus *196E.*

Compound	Concentration, *M*	Bacterial count after 30 min[a] ($\times 10^9$/ml)
None	--	1.5
Gramicidin	10^{-7}	0.00024
Valinomycin	10^{-5}	1.3
Monensin Na	10^{-5}	1.5
Lasalocid Na	10^{-5}	2.0
CCCP[b]	10^{-5}	1.5
DCCD[c]	10^{-5}	1.4
Ethanol control	d	1.3
None	--	2.1
Na_3VO_4	10^{-3}	0.0013
Tetraethylammonium chloride	10^{-3}	2.2
Quinine HCl	10^{-3}	<0.00001
Quinidine HCl	10^{-3}	2.0
Chlorpromazine HCl	5×10^{-5}	0.13
Tetracaine HCl	10^{-3}	<0.00001
Verapamil HCl	5×10^{-4}	2.9

[a]*S. aureus* cells were incubated in 0.1 *M* potassium phosphate buffer + 5% (w/v) NaCl, pH 7.5 held at 37°C.
[b]CCCP = carbonylcyanide m-chlorophenylhydrazone.
[c]DCCD = N,N'-dicyclohexylcarbodiimide.
[d]The ethanol concentration was approximately 750 μg.

Intact cells of lactose-grown *S. aureus* 196E will act on ONPG to release o-nitrophenol; however, if cellular integrity is lost (e.g., lysis), ONPG is not attacked. The actual substrate for the "ß-galactosidase" in *S. aureus* is the phosphorylated derivative of ONPG or lactose, and lysed cells can not phosphorylate these compounds unless phosphoenolpyruvate is added *(9)*. Thus the enzyme is really a phospho-ß-galactosidase. The differential effect of intact and lysed cells on ONPG or o-nitrophenyl-ß-galactosidase-6-phosphate (ONPG-P; the phosphorylated derivative does not appear to be permeable to intact cells) allows the opportunity to test NaCl on phospho-ß-galactosidase directly. The data presented in Table 5 indicate that the intact (untreated) cells of *S. aureus* 196E release o-nitrophenol from ONPG and the release is inhibited by NaCl. Low levels of o-nitrophenol were released from ONPG-P. This is likely due to action of cells on contaminating ONPG, and it appears that intact cells are not permeable to ONPG-P. Lysed cells (treated with lysostaphin) released essentially no o-nitrophenol from ONPG and the amount of o-nitrophenol released from ONPG-P was approximately the same with or without salt. Lack of activity with ONPG indicates that cell lysis was essentially complete (Table 5). Thus, NaCl does not appear to inhibit breakdown of ONPG in intact cells by adversely acting on phospho-ß-galactosidase, per se.

Sodium chloride, at 5% concentration, inhibited utilization of glucose (as measured by residual glucose in the medium) in *S. aureus* 196E by approximately 50% (Fig. 1). Data in Fig. 3 indicated that DCCD and the ionophores did not reverse the inhibitory effect of NaCl

TABLE 5. *The effect of sodium chloride on phospho-ß-galactosidase activity in intact and lysed cells of lactose-grown* S. aureus *196E.*

Cell preparation	NaCl in enzyme assay	Enzyme substrate ONPG[a]	Enzyme substrate ONPG-P[a]
Untreated	0.0%	306.9[b]	31.9
	5.0%	98.6	9.0
Treated with lysostaphin	0.0%	0.0	379.1
	5.0%	1.7	363.1

[a]ONPG = o-nitrophenyl-ß-galactoside (Sigma); ONPG = o-nitrophenyl-ß-galactoside phosphate (Sigma).
[b]Values represent μg o-nitrophenol released from ONPG or ONPG-P in 30 min using 10 mg dry weight cells.

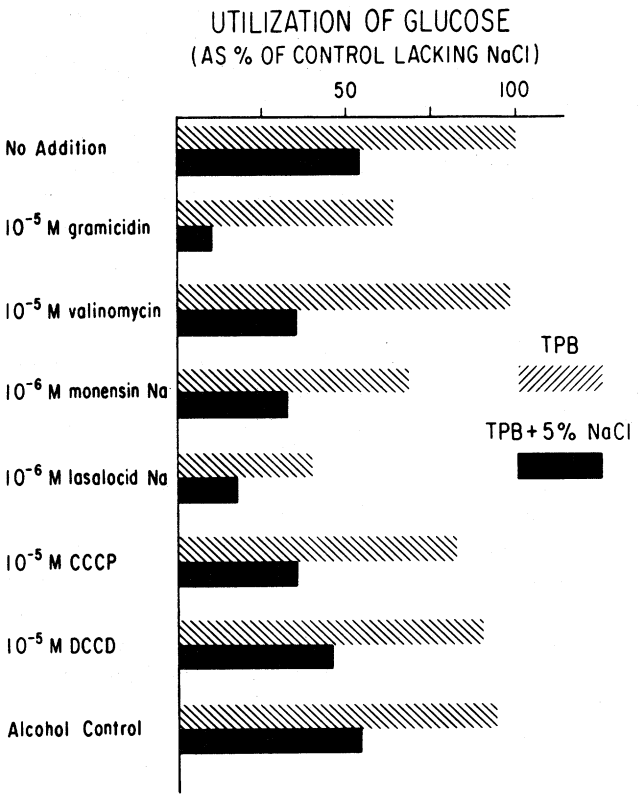


Figure 3. *Effect of ionophores and DCCD, in the presence or absence of 5% NaCl, on the utilization of glucose by* S. aureus *196E.*

on glucose utilization. In fact, most of the compounds potentiated the inhibitory effect of salt to some degree. Gramicidin, monensin, and lasalocid inhibited glucose utilization in the absence of NaCl. Na_3VO_4 and the ion channel blockers had no effect on glucose utilization in *S. aureus* 196E; there was neither reversal nor potentiation of NaCl inhibition (data not shown; the compounds were tested at the same levels given in Fig. 2B). The failure of the ionophores, ATPase inhibitors, and ion channel blockers to reverse NaCl inhibition of glucose utilization is completely unlike the results shown in Fig. 2A and 2B where most of these compounds reversed salt inhibition of phospho-ß-galactosidase activity.

TABLE 6. *Effect of monensin, in the presence or absence of NaCl, on the uptake of ^{14}C-2-deoxyglucose (2-DOG) in* S. aureus *196E.*

Addition	TSB w/o glucose + U-^{14}C-2-DOG[a]	
	No NaCl	+ 5% NaCl
None	102.3[b]	51.7
10^{-6} *M* monensin Na	38.2	6.3
Ethanol control	97.8	43.9

[a]Carrier was unlabeled 0.25% (w/v) 2-DOG.
[b]Values represent counts/min/50 mg dry weight cells.

The uptake of 2-deoxyglucose (2-DOG) by *S. aureus* has been demonstrated *(8)* but it was shown to be metabolized only to the 6-phosphate level. Because of its limited metabolism, 2-DOG is an ideal compound to use for substrate uptake studies in *S. aureus*. The data presented in Table 6 show that uptake of ^{14}C-labeled 2-DOG was inhibited by both NaCl and monensin and combination of the salt and ionophore was even more inhibitory to uptake of 2-DOG. Thus the results obtained with 2-DOG was very similar to that found with glucose utilization.

DISCUSSION

The growth of *S. aureus* 196E was less affected by NaCl than was certain other biochemical parameters such as SEA and phospho-ß-galactosidase synthesis and enzyme activity, and glucose utilization. Sodium chloride has been shown to affect the synthesis of certain staphylococcal enterotoxins more severely than it does growth. Using a SEB producing strain of *S. aureus* McLean et al. *(11)* demonstrated that toxin production was approximately 4 times more sensitive to 4% NaCl than was growth. Similarly, Genigeorgis and his co-workers *(6)* found that SEC production was approximately 2.5 times more sensitive to salt (4%) than was growth. However, Markus and Silverman *(12)* found that the amount of SEA produced per growth unit was approximately the same regardless of the concentration of salt (0 to 10%). Troller and Stinson *(25)* showed that growth (increase in cell protein) of *S. aureus* 196E (SEA producer) or C243 (SEB producer) was reduced by approximately 20% when the salt level was increased from 0 to 5.3%. SEA level, however, was unchanged by increasing the salt level but SEB production was decreased by more than 4-fold *(25)*. Pereira et al. *(14)*, using *S. aureus* S-6 which produced both SEA and SEB, found that as the salt level increased from 0 to 4% that there was only about 1.5-fold decrease in SEA production whereas there was a 6-fold decrease in SEB production. The inhibition of SEA synthesis by salt (4.1 times more sensitive than growth) shown in Fig. 1 is a contrast to the results obtained with SEA by Markus and Silverman *(12)*, Troller and Stinson *(25)*, or Pereira et al. *(14)*. However, those workers did their experiments under conditions in which cell numbers increased, whereas our studies were conducted under non-growth conditions.

The synthesis of phospho-ß-galactosidase in *S. aureus* 196E was 3.5 times more sensitive to NaCl than growth (Fig. 1). Similarly, Troller and Stinson *(25)* showed that the production of catalase was decreased approximately 4- and 14-fold with *S. aureus* strains 196E and C243, respectively, as the NaCl concentration increased from 0 to 5.3%, whereas growth (increase in cell protein) was decreased by only 20%. However, Troller and Stinson *(25)* found that the production of acid phosphatase was inhibited only 20% in *S. aureus* 196E while in strain C243, there was an actual increase in acid phosphatase production as the salt level increased. The synthesis of proteins, therefore, is not always inhibited by the addition of NaCl to *S. aureus* cultures.

Respiratory activity in *S. aureus* 196E was inhibited by NaCl. Other workers have shown that salt inhibits respiration in other microbial species. As the NaCl concentration increased from approximately 1 to 5%, Prior *(15)* showed that O_2 uptake ceased in *Pseudomonas fluorescens* when glucose, lactate, or arginine were the substrates. Erecinska and Deutsch *(5)*, increasing the NaCl concentration from 0.1 to 1.7%, found that there was a 2.7-fold decrease in O_2 uptake with glucose in *P. denitrificans*. Data presented here on salt inhibition of respiratory activity in *S. aureus* 196E as well as the results obtained by Prior *(15)* and Erecinska and Deutsch *(5)* suggest that NaCl inhibits utilization of various substrates either by interfering with transport of the compound into the cell or by inactivating intracellular enzymes involved in the metabolism of the substrates.

Sodium chloride inhibited the utilization of glucose (as measured by residual glucose in the medium) as well as inhibited the uptake of radiolabeled 2-DOG. Marsh et al. *(13)* using washed cells of *Streptococcus mutans* found that NaCl (approximately 0.8%) inhibited acid production when glucose or sucrose were the substrates. Marsh and his co-workers *(13)* felt that energy dissipation due to the exclusion of sodium ions from the cell prevented the transport of sugars. Woods and Wood *(26)* demonstrated the progressive inhibition of glucose utilization and progressive decrease in intracellular ATP level with increasing concentrations of NaCl (0.5 to 5.5%). The per cent conversion of glucose into ethanol remained constant regardless of NaCl levels. Thus NaCl interfered with glucose uptake but not with its metabolism *(26)*. Using α-aminoisobutyric acid, a non-metabolizable amino acid, Erecinska and Deutsch *(5)* showed that increasing the NaCl levels from 0.2 to 1.8% inhibited the uptake of the amino acid completely in *P. denitrificans*. Our results showing NaCl inhibition of glucose utilization and prevention of 2-DOG uptake and that of Marsh et al. *(13)*, Woods and Wood *(26)*, and Erecinska and Deutsch *(5)* support the hypothesis that NaCl acts by preventing the entry of substrates into the cell possibly by the dissipation of energy used to exclude Na^+ from the cell rather than being used to transport substrates.

A number of ionophores, ATPase inhibitors, and ion channel blockers were effective in reversing the NaCl in-

hibition of ONPG breakdown by lactose-grown *S. aureus* 196E and many of the compounds stimulated ONPG uptake in the absence of salt. However, these results were confusing since the ionophores, ATPase inhibitors, and ion channel blockers did not reverse sodium chloride inhibition of glucose utilization in *S. aureus* 196E and none of them stimulated glucose utilization in the absence of salt. Our data indicate that ionophores, ATPase inhibitors, or ion channel blockers do not have predictable effects on substrate uptake or utilization in *S. aureus* 196E.

Other workers have shown that ionophores and DCCD inhibit the uptake of substrate in a number of microbial systems. Thompson *(23)* showed that the proton-conducting uncoupler, CCCP, inhibited galactose uptake by APT-permease in *Streptococcus lactis;* however, the ionophore had no effect on galactose transport mediated via the phosphoenolpyruvate phosphotransferase system in the same organism. Similarly, galactose uptake in *S. thermophilus* was inhibited by CCCP; however, DCCD had no effect *(7)*. Kitada and Horikoshi *(10)*, using alkalophilic *Bacillus* no. 8-1, found that α-aminoisobutyrate uptake was inhibited and the intracelluar ATP level was decreased by the addition of CCCP, valinomycin, or monensin. The transport of 4-nitrophenyl-D-glucoside was inhibited by DCCD and CCCP in intact cells of *Saccharomyces cerevisiae* and *Candida utilis;* from these data, Sims et al. *(17)* suggested that transport of the glucose analogue involved proton symport, with ATPase activity maintaining the proton gradient. Cason et al. *(1)* showed that *S. cerevisiae* transported the non-metabolizable sugar, L-sorbose, via proton symport and the transport was inhibited by CCCP.

In contrast to the work presented by Thompson *(23)*, Hutkins et al. *(7)*, Kitada and Horikoshi *(10)*, Sims et al. *(17)*, and Cason et al. *(1)*, where uptake of substrates was prevented by the addition of ionophores and/or DCCD, Singh and his coworkers *(19)*, utilizing *Brochothrix thermosphacta* obtained stimulation of the uptake of α-methylglucoside by the addition of CCCP or valinomycin. Additionally, Singh et al. *(19)* showed stimulation of 2-DOG and glucose uptake with CCCP addition.

Sodium chloride inhibited hydrolysis of ONPG by intact cells of *S. aureus* 196E but salt did not inhibit the action of phospho-ß-galactosidase per se (Table 5); thus salt seems to inhibit the transport of the ß-galactoside. Ionophores, DCCD, and ion channel blockers reversed the inhibition of ONPG uptake by NaCl by an effect which appears to be an increase in cell permeability in *S. aureus*. Rosen *(16)*, using *E. coli* NR70 (a mutant lacking Mg^{++}-ATPase), showed that DCCD stimulated the uptake of thio-methyl-ß-galactoside. Rosen suggested that DCCD reduced proton release from the cells and at the same time, increased ß-galactoside transport. Singh and Bragg *(18)* demonstrated that the addition of DCCD to *E. coli* NSl (a lipopolysaccharide-deficient mutant) led to an increase in the hydrolysis of ONPG. There was no corresponding increase in the leakage of ultraviolet absorbing materials or free ß-galactosidase into the medium. Lysis of the *E. coli* strain was not observed when DCCD was present *(18)*. Thus DCCD appears to increase the permeability of ONPG to *E. coli* NSl. Our results suggest that ionophores, DCCD, and ion channel blockers increase the permeability of ONPG to *S. aureus* 196E when entry of that substrate was blocked by NaCl. However, these compounds did not reverse salt inhibition of glucose utilization, nor did monensin reverse salt inhibition of 2-DOG uptake. In *S. aureus* 196E, sodium chloride inhibited phospho-β-galactosidase synthesis with lactose as the inducer; addition of monensin did not overcome the salt inhibition of enzyme synthesis. Thus reversing the salt inhibition on ONPG hydrolysis by ionophores, DCCD, and ion channel blockers is probably due to some effect other than increased permeability.

Sodium chloride inhibition of respiration, ONPG hydrolysis, glucose utilization, and C^{14}-2-DOG uptake suggest that it prevents transport of substrate into the cells of *S. aureus* 196E. This suggestion is strengthened by the observation that lysed cells of lactose-grown *S. aureus* hydrolyzed ONPG-P equally well in the presence or absence of salt. For ONPG to be hydrolzyed by the enzyme, phospho-ß-galactosidase, it must first enter the cell and be phosphorylated via the phosphoenolpyruvate mediated phosphotransferase system. Since NaCl does not inhibit the hydrolysis of ONPG-P, the inhibitory effect of salt must be on the entry of ONPG into the cell. It is not clear how NaCl inhibits substrate entry into the cell but it is possible that the cell wastes energy by excluding sodium ions and thus less energy is available for transport of substrate across the cell membrane.

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